

In Vitro Screening of *Falcataria moluccana* (Miq.) with Gall Rust (*Uromycladium tepperianum* (Sacc.) Filtrate as Media Selection

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Abstract

In vitro screening of *Falcataria moluccana* (Miq.) was conducted by tissue culture method. Seeds from two different site of community forest, 400 m (S1) and 800 m (S2) above sea level, were used as material. Double concentration of MS (Murashige & Skoog, 1962) with 40 mg/l gall rust (*Uromycladium tepperianum* (Sacc.) filtrate were used for media selection. The results of this research showed that 66 % axenic plantlets in vitro from S1 and 27 % from S2 were still survived after 3 months incubation without subculture. The mean of fresh weight (2.21 ± 0.26 g) and dry weight (1.97 ± 0.12 g) from S1 plantlets lower than the mean of fresh weight (2.87 ± 0.18 g) and dry weight (2.16 ± 0.14 g) from S2 plantlets. Qualitative of terpenes, saponins and quantitative of total phenolics were analyzed from those gall rust extract, as source of filtrate media, attacked and un-attacked of *F. moluccana*. They all qualitatively have capability to produce terpenoid and saponin. It is notice that *U. tepperianum*, un-cultured pathogen, contain of those compound that may play a role as co-determinants of pathogenicity. While the highest total phenolic compound were contained in gall rust extract (2.35 %), followed by attacked *F. moluccana* branches (1.18 %) and un-attacked *F. moluccana* branches (0.44 %). This indicated that phenolic compound in gall rust has higher activity as a response of *F. moluccana* to *U. tepperianum* pathogen pressures and result of this study suggest the great value of gall rust filtrate for use as media selection in vitro.

Keywords : *F. moluccana*, *U. tepperianum*, gall rust, in vitro selection.

Introduction

The use of disease-resistance/tolerance plants eliminates the need for additional efforts to reduce disease losses unless other diseases are additionally present. Resistance plants are usually derived by standard breeding procedures of selection (Fry, 1982; Arneson, 2001; Maloy, 2005). Conventional tree breeding methods are often constrained by the long reproductive cycles

and by the difficulty in achieving significant improvements to complex traits such as disease (Nehra *et al.* 2005). Biotechnology has a potential to solve these problems. However, as base method, only a few reports of tissue culture in *F. moluccana* are available (Bon *et al.*, 1998; Sukartiningsih *et al.*, 2002; Sumiasri *et al.*, 2006).

Various approaches *in vitro* selection for disease resistance has been reviewed by van den Bulk (1991). *In vitro* selection has high effectiveness and efficiency to get the desired genetic characters with relative shorter time (Meredith, 1978; Watmough & Hutchinson, 1997; Gori *et al.*, 1998; van Sint *et al.*, 1997; Baraka *et al.*, 2011). Chemical compounds

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for *in vitro* selection is an activator media which induce plant resistance response known as systemic acquired resistance (SAR) and induced resistance (Maloy, 2005). *In vitro* selection seems to hold promise for increasing the mutation frequency and improvement in the methods of selection as somaclonal variation (Maloy & Baudoin, 2001; Maloy, 2005).

In their nature, plants protect themselves against pathogens by producing of low-molecular-weight antimicrobial molecules called secondary metabolites. Plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics and N with S containing compounds (Mazid *et al.*, 2011). A recent literature surveys, fungal have metabolites also, which examined 1,500 compounds that were isolated and characterized between 1993 and 2001. Many of these molecules had cytotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive, enzyme inhibitory, allelopathic and other biological effects also have been found (Keller *et al.*, 2005).

Gall formation represents the most intimate and specialized form of plant-pathogen interaction, and causes anatomical and metabolic alterations in the host plant functions (Edwards & Wratten 1980; Fernandes & Price 1988; Hartley 1998; Schönrogge *et al.* 2000; Moura *et al.* 2008; Formiga *et al.* 2009). Until now, phenolic compounds in non-galled tissues have been associated to a defensive plant mechanism against the gall inducers (Abrahamson *et al.* 1991; Hartley 1998; Nyman & Julkunen-Titto 2000; Pascual-Alvarado *et al.* 2008; Formiga *et al.* 2009). Previous histochemical studies found phenolic compounds in the cortex of both types of galls on *C. brevipes* (Carvalho *et al.* 2005). Gall rust disease caused by *Uromykladium tepperianum* (Sacc.) is one of the most destructive diseases in *F. moluccana* plantations (Rahayu *et al.*, 2008). *F. moluccana* - gall rust need to be analyzed to determine the content of the secondary metabolites

that may use to *in vitro* selection media for gall rust tolerant plant or by a wide range of potential pathogens.

U. tepperianum is an obligate biotrophs and not culturable *in vitro* so the gall can be used as modified fungal culture for *in vitro* selection. Gall filtrates may contain a spectrum of secondary metabolites, along with toxins that may play a role as co-determinants of pathogenecity during disease development (Svabova & Lebeda, 2005). Toxin were found in various filtrates of the two main types of plant-pathogen interactions, i.e., host-selective and non-host-selective. The application of filtrat to cultures *in vitro* can trigger the elicitation of various defence responses, e.g., phytoalexins; activity of certain enzymes (Lebeda *et al.*, 2001); total phenol, peroxidases and beta 1,3-glucanase (Lebeda *et al.*, 2001; Singh *et al.*, 2003); and chitinase (Jayasankar & Litz, 1998).

Selection of disease tolerance *in vitro* is achieved by exposing a large number of cells to a selective agent such as a pathogen or phytotoxin and subsequent regeneration of plant from the surviving cells. These surviving cells may have been present as genetic variants within the explants or they may be somaclonal variants that have arisen spontaneously *in vitro*. Selection does not necessarily have to be carried out at the cellular level. In fact there may be drawbacks in doing so because the response of cells *in vitro* may be different from that of plants in the field (Sharma & Skidmore, 1988). Therefore, it is sometimes better to carry out the selection *in vitro* with micropropagated shoots rather than with cells (Bonga & Aderkas, 1992).

Dependent on local climate, which is affected by the altitude varies above sea level, relative humidity and wind speed, were meteorological factors which were significantly associated with the incidence and severity of gall rust disease. High relative humidity (RH \geq 90%) and slow wind speed (WS \leq 80 km/hours), was found to promote gall rust disease development (Rahayu, 2007).

High risk of diseases also due to fog, one of the most significant factors supporting gall rust disease development (Rahayu, 2011).

Only a few reports on tissue culture in *F. moluccana* are available (Bon *et al.*, 1998; Sukarutiningsih *et al.*, 2002; Sumiasri *et al.*, 2006). In our previous research reported that plantlets were successfully regenerated from nodes obtained from *F. moluccana* seedlings, when these explants were cultured on a 2MS (Murashige & Skoog, 1962) medium with BAP (6-benzylaminopurine) and NAA (Naphthalene Acetic Acid) at 25°C under 16-hours photoperiod (Putri, 2013). In the present study an attempt was made to establishment of previous research of *F. moluccana* tissue culture technique as basic principles for in vitro selections and also to prove crude gall rust filtrate may used as tissue culture selective agents in *F. moluccana* tolerant studies. However, tissue culture techniques should be developed in this species for diseases tolerance selection.

Material and Methods

Plant and gall rust material

F. moluccana bulk seeds which used to in vitro selection were collected from 5 un-attacked gall rust trees at 800 m asl. (S2). Material for extraction and analyzed were collected from 10 gall rust, 10 branches from 5 un-attacked trees and 10 branches from attacked trees. Un-attacked trees were determined by not any gall rust grew on all parts of the the plant. These material collected from community forest at Kaliurang, Yogyakarta at 400 m asl. (S1) and community forest at Blitar, East Java at 800 m asl. (S2).

In vitro selection with tissue culture techniques

The basal media for *F. moluccana* regeneration in tissue culture (Putri, 2013) were used for this selection. Seeds from un-attacked plant were sterilized with 70% ethylalcohol for 30 second, and then for 30 minutes with 0.15 % sublimat (HgCl₂) before planted in basal media. In our previous research, the filtrate dose level that killed 50

% (LD 50) until 1 month observation on *F. moluccana* plantlets was in 2MS with gall rust 40 mg/l filtrat added (25 % filtrat solution) (Putri, 2014). This research was to knew about the range of dose level (from 1 mg/l until 100 mg/l filtrat added), because not any report about this before. In present research, the filtrate concentration was used as tolerant selection media. 100 axenic plantlets will be transferred to this selection media and observed until 3 months without subculture. Culture without filtrate added observed as a control. The effect of the selection agent in vitro can be accessed via various parameters, e.g., inhibition of the culture weight growth, percentage of regenerating explants, percentage of necrotic explants, colour or morphological changes (Hollmann *et al.*, 2002).

This observation research based on un-dead axenic plantlets on media selection and weight growth of plantlets. Effects on *F. moluccana* growth were observed as fresh and dry weight of un-dead plantlets. The culture were incubated under light conditions (16 hours day) and maintained at 22 °C - 25 °C temperature with 60 % - 70 % humidity until 3 months.

Extraction of U. tepperianum

Kinetic maceration method was used in extraction of *U. tepperianum*. Powdered *U. tepperianum* was mixed thoroughly with 95 % ethanol, extracted by shaking for about 30 minutes, leaving the mixture for 24 hours and than filtered. The process was repeated for 3 times. The filtrate was evaporated with vacuum rotary evaporator at 70°C water bath. Finally the viscous extract was placed at porcelain plate, continuously steered and heated at 70°C water bath. Extract was diluted with dimethyle sulfoxide (Wagner *et al.*, 1984).

Qualitative analysis of saponin and terpene

Thin layer chromatography (TLC) test was used for terpenes and saponins qualitative

analysis. Gall rust, infected branch and uninfected branch material (50 mg each) were mixed thoroughly with 1 ml of methanol, then rotating with vortex for 2 minutes. Centrifuge for 3 minutes, then spotting 20 μ l samples at 60 F254 silica gel plate, with β -sitosterol as reference solution. For analysis of steroid, put the sample into a chamber with saturated mobile phase of hexan:ethylacetat (70:30) and eluation up to chamber line, lift and drain; than spray with lieberman bucard reagent then heat on 110^oC for 2 minutes. For analysis of saponin, put the sample into a chamber with saturated mobile phase of Chloroform:methanol (90:5) and luation up to chamber line, lift and drain than spray with anisaldehyde sulfidic acid reagent. For analysis of terpene, put the sample into a chamber with saturated mobile phase of tholuene: ethile (93:7) and eluation up to chamber line, lift and drain; than spray with vanillin sulfidic acid reagent (Wagner *et al.*, 1984).

Quantitatif analysis of total phenolic

Aliquots of the extracts were taken in a 10 ml glass tube and made up to a volume of 3 ml with distilled water. Then 0.5 ml folin ciocalteau reagent (1:1 with water) and 2 ml Na₂CO₃ (20%) were added sequentially in each tube. A blue color was developed in each tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in folin ciocalteau reagent in alkaline medium which resulted in a blue colored complex, molybdenum blue. The test solutions were warmed for 1 minute, cooled and absorbance was measured at 650 nm against the reagent used as a blank. A standard calibration plot was generated. The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample (Malik & Singh, 1980).

Results and Discussion

In vitro screening of *F. moluccana* (Miq.) was determined by seeds from S1 (400 m asl.) and S2 (800 m asl.) forest. Data presented in

Table 1 showed that percent of survive of S1 higher than S2. 27 % of plantlet at S2 which have more humid and foggy than S1 may lead their adaptation to climate conditions. Adaptation of anatomical and morphological properties such as impermeable cuticles and intracellular modification are thus more resistance to gall rust fungus infections (Rahayu, 2011). The application of pathogen filtrat to cultures in vitro can trigger the elicitation of various secondary metabolites as defence responses (Jayasankar & Litz, 1998; Lebeda *et al.*, 2001; Singh *et al.*, 2003).

Table 1. In vitro screening of *F. moluccana*

Plantlets	Survive (%)	Mean(\pm SE)* of fresh weight	Mean(\pm SE)* of dry weight
S1	66	2. 21 \pm 0,26	1. 97 \pm 0. 12
S2	27	2. 87 \pm 0,18	2. 16 \pm 0. 14
Control	100	2. 94 \pm 0,34	2. 18 \pm 0. 17

* \pm SE mean values were calculated from 25 replication

The mean of fresh weight (2. 21 \pm 0. 26 g) and dry weight (1. 97 \pm 0. 12 g) from S1 plantlets lower than the mean of fresh weight (2. 87 \pm 0,18 g) and dry weight (2. 16 \pm 0. 14 g) from S2 plantlets. Although higher concentrations of secondary metabolites might result in a more resistant plant, the production of secondary metabolites is thought to be costly and reduces plant growth and reproduction (Simms, 1992; Karban & Baldwin, 1999; Simens *et al.*, 2002). The lower fresh and dry weight of S1 than S2 can be caused by higher energy for durability, higher secondary metabolites as defence responses and reduces of growth. The cost of defense has also been invoked to explain why plants have evolved induced defense, where concentrations generally increase only in stress situations (Harvell & Tollrian, 1999). The growth of *F. moluccana* plantlets were tested in media selection (Figure 1). Tolerant planlets in this media would be inhibited in root growth but not decayed, while un-tolerant plantlets were decayed. Plants produce a high diversity of secondary metabolites including terpenes, saponins and phenolic compounds

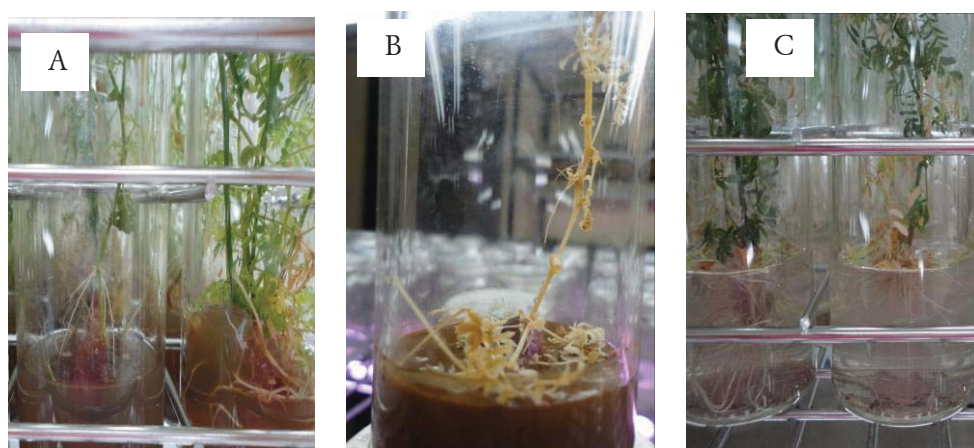


Figure 1. In vitro screening of *F. moluccana* (Miq.) with gall rust (*U. tepperianum* (Sacc.) filtrate) as media selection: tolerant plantlets (A), un-tolerant plantlets (B) and control (C) after 3 months incubation.

with a prominent function in the protection mechanisms against pathogens on the basis of their toxic nature (Schafer *et al.*, 2009), and are insignificant for growth and developmental processes (Rosenthal *et al.*, 1991). The ability of plants to experience damage without a reduction in growth and development fitness will be used as originally defined of tolerance by Rausher (1992).

By their nature, plants protect themselves by producing some compounds called as secondary metabolites. There are three major groups of secondary metabolites viz terpenes, phenolics and N and S containing compounds (include saponins), defend plants against a variety of pathogenic microorganisms (Rosenthal *et al.*, 1992; van Etten *et al.*, 2001). In this research, analysis of terpenes, saponins and phenolic showed that all of gall rust extract, attacked and un-attacked of *F. moluccana* have capability to produce terpenoid, saponin and phenolic compound (Table 2).

Table 2. Capability of *F. moluccana* to produce terpenoid, saponin and phenolic

No.	Material	Terpene	Saponin	Total Phenolic
1.	Gall rust	+	+	2.35 %
2.	attacked	+	+	1.18 %
3.	un-attacked	+	+	0.44 %

+: capable

This results except phenolic analysis can be showed from thin layer chromatography which can detected on silica gel 60 F254 as adsorbent (Figure 2).

Vaniline sulfidic acid detection for terpenes showed grey bands on preparative silica gel plates under visible light, indicating the presence of terpenes (2.A). Every compound have a special retention value (R_f) for every specific solvent and solvent concentration. This value can identification of unknown sample or its constituent with R_f values of known compounds (Erdman *et al.*, 1990). R_f values terpenes were 0.67 for un-attacked trees and gall rust; 0.64 for attacked trees. Terpenes constitute the largest class of secondary metabolites and are united by their common biosynthetic origin from acetyl-coA or glycolytic intermediates (Gershenzon *et al.*, 1991; Grayson, 1998; Fraga, 1988; Croteas, 1988; Loomis & Croteas, 1980; Robinson, 1980). A vast majority of the different terpenes structures produced by plants as secondary metabolites that are presumed to be involved in defense as toxins and feeding deterrents to a large number of plant feeding insects and mammals (Gershenzon & Croteau, 1991).

Anisaldehyde sulfidic acid detection for saponins showed brown bands on preparative silica gel plates under visible light, indicating the presence of saponins (2.B). R_f values

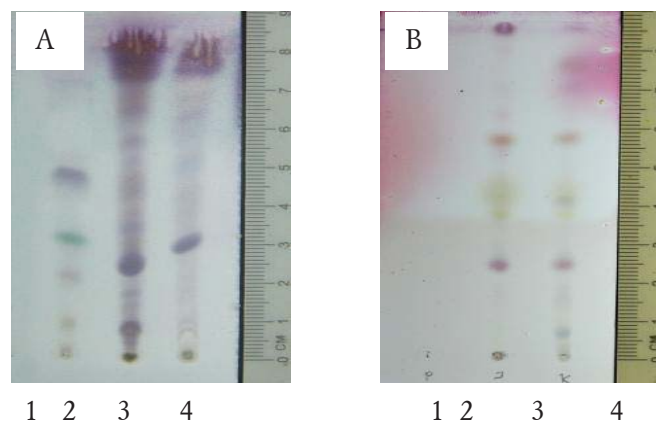


Figure 2. Analysis of terpenoid (A) and saponin (B) (all spots) from *F. moluccana* un-attacked (2), *F. moluccana* attacked (3) and gall rust (4) with quinine comparator (1), by thin layer chromatography, separation with visible light observation.

saponins were 0.36 for un-attacked trees; 0.29 for gall rust and 0.56 for attacked trees. R_f values are between 0 and 1 and best between 0.1 and 0.8; a parameter often used for qualitative evaluation. Saponins are secondary plant metabolites that occur in a wide range of plant species (Hostettmann & Marston, 1995). They are stored in plant cells as inactive precursors but are readily converted into biologically active antibiotics by plant enzymes in response to pathogen attack. These compounds can also be regarded as preformed, since the plant enzymes that activate them are already present in healthy plant tissues (Osborn, 1996). The natural role of saponins in plants is thought to be protection against attack by pathogens (Price *et al.* 1987; Morrissey & Osborn, 1999).

Alcaloids, another N and S containing compounds secondary metabolite, were not presented on preparative silica gel plates under visible light in Dragendorff detection of *F. moluccana* (Putri, 2014). Thousands of different alkaloids have been discovered from throughout the plant kingdom, but there are some species that do not contain any of these bitter, nitrogenous compounds. Lieberman Bucard detection for *F. moluccana* steroid showed brown bands on preparative silica gel plates under visible light, indicating the presence of steroids (Putri, 2014).

A clear and broader understanding of the *F. moluccana* - *U. tepperianum* secondary metabolites have not been fully investigated in all aspects, for it would open up possibilities of developing novel, more effective and sustainable strategies to control or eradicate fungal diseases in plants.

In vitro culture of plant cells, tissues or organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance which includes the use of some selective agents that permit the preferential survival and growth of desired phenotypes (Purohit *et al.*, 1998). The selecting agents usually employed for in vitro selection include specific fungal culture filtrate (FCF) or phytotoxin such as fusaric acid or the pathogen itself (for disease-resistance) (Raja *et al.*, 2011). This preliminary research focuses on the progress made towards the development of *F. moluccana* tolerant lines in *U. tepperianum* extract that have specific steroidal toxin through tissue culture based in vitro selection.

Conclusion

The study revealed that regeneration with tissue culture was potential alternative technique for trees disease tolerant selection.

Falcataria moluccana (Miq.) tissue culture regeneration medium with gall rust (*Uromycladium tepperianum* (Sacc.) filtrate added may use for media selection. The implication of this *in vitro* selection study with tissue culture is very crucial as it provided tolerant species and regenerate *in vitro* nature for tree improvement purpose. Since gall rust disease caused by *Uromycladium tepperianum* (Sacc.) is one of the most destructive diseases in *F. moluccana* plantations, they need *in vitro* full control environment to know what exactly medium for their develop and need to be analyzed to determine the content of the secondary metabolites that may use to *in vitro* selection media for gall rust tolerant. With the results obtained in this study, it is therefore recommended to continue observation, so that it can be understand more clearly.

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